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Virology 350 (2006) 381–393

VIROLOGY

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The Epstein–Barr virus oncoprotein LMP1 inhibits the activity of viral or cellular promoters without inducing cytostasis

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Received 20 December 2005; returned to author for revision 10 January 2006; accepted 8 February 2006
Available online 13 March 2006

Abstract

The Latent Membrane Protein 1 of the Epstein–Barr virus is required for human B lymphocyte immortalization and functions as a constitutively activated member of the TNF-receptor family, through recruitment of TRAFs and TRADD molecules on its Carboxy-terminal domain, leading to the activation of NF-κB and AP1 transcription factors. The formation of the signaling complexes requires LMP1 oligomerization, a role assigned to the membrane-spanning domains of the molecule. There is, however, increasing evidence that these membrane-spanning domains are not only confined to oligomerization but play a direct role in downregulation of promoter activity and cytostasis. Here, we describe a new inhibitory activity which is effective on viral or cellular promoters (even the endogenous ones), requires only membrane-spanning domains 3–4 or 5–6 and is neither associated with cytostasis nor with apoptosis.

Keywords: EBV; LMP1; Membrane-spanning domains; Promoters; Cytostasis

Introduction

Epstein–Barr virus (EBV) is an ubiquitous B-lymphotropic γ-herpes virus which is thought to latently infect more than 95% of the world's adult population. EBV is the causative agent of infectious mononucleosis but is also associated with several malignant diseases of epithelial or lymphoid origin including Burkitt's lymphoma (BL), Hodgkin's disease (HD), T cell lymphoma, post-transplant lymphoproliferative disorders, gastric carcinoma and undifferentiated nasopharyngeal carcinoma (NPC) (Young and Rickinson, 2004). In vitro, EBV efficiently infects human B lymphocytes and confers them the capacity to grow under culture conditions for a greatly extended period of time, during which some will become truly immortalized lymphoblastoid cell lines (LCL) by reactivating cellular functions, particularly telomerase activity (Sugimoto et al., 2004). Despite the fact that the viral genes necessary for in vitro B lymphocyte immortalization have been identified (Kieff and Rickinson, 2001), the role of EBV in malignant transformation is far from being understood and very likely varies from one tumor to the next, as does the pattern of viral gene expression. However, analysis of the transforming potential of viral genes expressed in different tumors early identified the BNLF1 gene as an oncogene, as its ectopic expression in rodent cells induced their malignant transformation and conferred them the ability to form tumors in nude mice (Baichwal and Sugden, 1988; Wang et al., 1985). Moreover, transgenic mice expressing the BNLF1 gene in skin or in B lymphocytes develop skin hyperplasia (Wilson et al., 1990) and B lymphoma, respectively (Kulwichit et al., 1998). The BNLF1 gene thus displays all the characteristics of an oncogene and is expressed in several types of latency to produce an integral membrane protein: LMP1. LMP1 is composed of cytoplasmic N terminal and C terminal domains (respectively 24 and 200 amino acids long in the B95-8 strain), separated by 160 amino acids divided between 6 hydrophobic transmembrane (TM) segments connected by short extramembrane loops (Eliopoulos and Rickinson, 1998). LMP1 expression in epithelial or lymphoid cells results in upregulation of numerous cellular proteins, including MHC class II molecules (Zhang et al., 1994), adhesion molecules such as ICAM1/CD54 (Wang et al.,

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doi:10.1016/j.virol.2006.02.010

1988), proteins with anti-apoptotic activity such as Bcl2 (Henderson et al., 1991) and A20 (Laherty et al., 1992), receptors such as EGF-R (Miller et al., 1995), and many other factors involved in tumor metastasis such as cyclooxygenase-2 (Murono et al., 2001) and metalloproteinases (Takeshita et al., 1999). LMP1 spontaneously oligomerizes in membrane lipid rafts, which appears to be necessary for the LMP1 C-terminal domain to recruit cellular factors normally associated to the TNF-α receptor (TRAFs) and other members of this family (IL-1R, CD30, CD40). This recruitment is mediated by the C-terminal Activating Regions (CTARs), either directly via the CTAR1 located just proximal to the cell membrane (amino acids 194–231) or via the TNF receptor-associated death domain (TRADD) which binds to the CTAR2 (corresponding to C-terminal amino acids 351–386) (Eliopoulos and Young, 2001). The recruitment of TRAFs, which are normally associated with molecules of the TNF-R family, suggests that LMP1 mimics a ligand-independent, constitutively activated receptor functionally related to this family (Lam and Sugden, 2003). The demonstration that intracellular C-terminal regions of LMP1 and CD40 are interchangeable is evidence that they belong to the same functional family despite the fact that they share very little protein homology (Gires et al., 1997). The binding of TRAFs (and possibly other as yet unidentified factors) to LMP1 initiates cascades of phosphorylations resulting in the activation of numerous transcription factors including NF-κB (Hammarskjold and Simurda, 1992), AP-1 (Kieser et al., 1997), ATF2 (Eliopoulos et al., 1999) and certain STATs (Chen et al., 2003; Gires et al., 1999), which likely accounts for all the described LMP1 induced cellular modifications.

In contrast with these previous observations, it has been more recently described that LMP1 is able to inhibit the activity of several EBV promoters, including its own promoter and EBNA Cp. It is probable that numerous cellular promoters are sensitive to this inhibition, as LMP1 expression was noted to significantly decrease cellular protein synthesis (Sandberg et al., 2000). It was hypothesized that this general inhibition could be the necessary counterpart allowing the cell to overcome the wide activating properties of LMP1, which in the long term could be heavily detrimental. This LMP1-dependent inhibition of protein synthesis also offers a very logical explanation for the reported toxicity of overexpressed LMP1 (Hammerschmidt et al., 1989), and for more recent reports indicating that LMP1 expression blocks cell division and induces long-lasting cytostasis (Coffin et al., 2001, 2003; Floettmann et al., 1996; Kaykas and Sugden, 2000). However, these later observations are difficult to reconcile with the oncogenic properties of LMP1, whose expression has been widely documented in fast-growing tumors, including the vast majority of EBV positive HD and NPC (Kieff and Rickinson, 2001). Moreover, an elegant study of Kilger et al. (1998) suggests that LMP1 is necessary for the proliferation of EBV-immortalized B lymphocytes, in which it could play the same role as the activated CD40 receptor. The reasons for these observed opposite effects of LMP1 expression are not clear and the use of different cellular models does not account for all the observed discrepancies, as conflicting results have been obtained by Floettmann et al. (1996) using the DG75 B cell line. LMP1 thus appears to be a very complex regulator able to activate numerous signaling pathways and to concomitantly inhibit gene expression from a large body of cellular promoters.

These paradoxical effects prompted us to investigate the effects of LMP1 on various natural complex promoters in transiently transfected DG75 cells. We chose to focus on widely used ubiquitous viral or cellular promoters believed to be expressed in most cells, to be poorly sensitive to the cellular context and containing *cis*-acting elements for transcription factors activated by LMP1: the immediate early promoter of the human cytomegalovirus (IE-CMV) (Lee et al., 2004), the early promoter of the Simian Virus 40 (E-SV40) (Tabakin-Fix et al., 2004), the promoter controlling the expression of the protein elongation factor 1α gene (EF-1α) (Wakabayashi-Ito and Nagata, 1994) and the promoter of the Glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH). In this paper, we report that LMP1 inhibits the activity of several (but not all) promoters of viral or cellular origin and that a mutant LMP1 lacking the C-terminal region retains the full inhibitory capacity of the wild-type protein. Using a battery of mutants with various combinations of TM domains, we were able to map the involved region to the TM domains 3–4 and 5–6, without implication of the TM domains 1–2. Interestingly, we could exclude the trivial hypothesis that this LMP1-induced inhibition resulted from apoptosis of the transfected cells. Moreover, under our conditions leading to inhibition of gene expression, LMP1 does not induce cytostasis and LMP1-positive DG75 cells continued to proliferate at the same rate as cells transfected with a control gene. We thereby describe a possibly new effect of LMP1, inhibiting gene expression from some but not all promoters, without affecting cell viability or proliferation.

Results

LMP1 inhibits the activity of some but not all promoters

For the sake of simplicity and according to Sandberg et al., we define promoter activity as the sum of all cellular processes required to generate a reporter signal in our transfection experiments. It has been shown that LMP1 was able to inhibit gene expression from the EBV *Bam-C* promoter or from the BNLF1 gene promoter and that this inhibition was associated with a general decrease in protein synthesis in BJAB cells (Sandberg et al., 2000). As LMP1 is also known to activate several transcription factors, it was of interest to study its effect on natural complex promoters containing response elements for different transcription factors, some of which able to be activated by LMP1. Promoters from viral (IE-CMV) or cellular (EF-1α, GAPDH) origins were cloned in front of the firefly luciferase gene to generate pCin-Luc, pEF-Luc or pGAPDH-Luc, respectively. The pGL3 vector (Promega), containing the same luciferase gene, was used to test the E-SV40 promoter. These constructs were independently cotransfected in DG75 cells with increasing amounts of an expression vector for the B95-8 BNLF1 gene (pCin-LMP1) and the empty vector pCi-neo was introduced to equalize plasmid quantities in all

experiments. As a control, we used a plasmid similar to pCin-LMP1 but expressing the EGFP gene instead of the BNLF1 gene (pEGFP-N3, Clontech). This choice resulted from preliminary observations indicating that different expression vectors, regardless of the gene they contained, partially inhibited in a nonspecific way luciferase production when cotransfected with the luciferase expression vector pCin-Luc in DG75 cells (Fig. 1A). This inhibition, which was never observed when an empty vector was cotransfected with pCin-Luc, likely results from competition between the plasmids or their derived mRNAs for transcription and/or translation factors present in limiting concentrations. However, in addition to the non specific inhibition observed following expression of any gene, LMP1 always induced a stronger and specific inhibition of luciferase production (Fig. 1A). In order to only evaluate this specific LMP1-induced inhibition in all subsequent experiments, the luciferase activity measured in the presence of increasing amounts of the expression vectors for LMP1 or its derivatives was normalized against the luciferase activity detected in the presence of identical quantities of an expression plasmid for a neutral gene (EGFP or β-galactosidase), considered as 100%. In these conditions, the effect of increasing amounts of LMP1 on various promoters was analyzed and is shown in Figs. 1B, C and D. Interestingly, LMP1 induced a strong and specific inhibition of the IE-CMV and EF-1α promoters (and of the GAPDH promoter, result not shown) when the E-SV40 promoter appeared completely insensitive to this inhibition. In order to make sure that this inhibition did not result from unexpected effects possibly induced by large quantities of LMP1, we verified the activation of the NF-κB signaling pathway using the pNF-κB-Luc reporter plasmid, containing 4 NF-κB binding sites originating from the κ light chain enhancer element. As expected, the largest quantity (4 μg) of pCin-LMP used in the previous tests induced a robust and specific 10-fold increase of the luciferase activity over the controls (Fig. 1E). Using Western blot analysis, we also verified

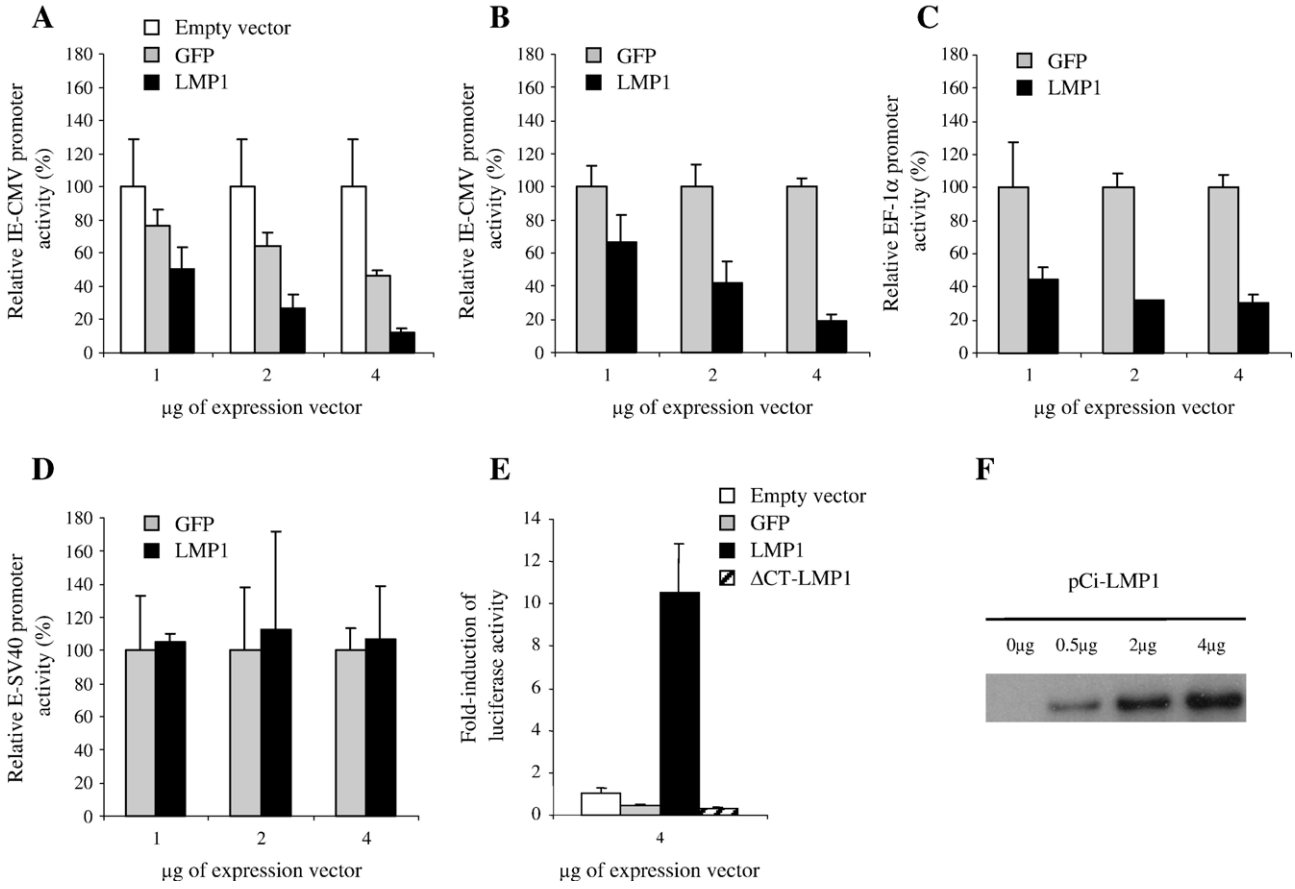

Fig. 1. Effect of LMP1 on the activity of the IE-CMV, E-SV40 and EF-1α promoters. (A) One million DG75 cells were electroporated in the presence of 2 μg pCin-Luc vector and the indicated quantities of pCi-neo (open bars), pEGFP-N3 (grey bars) or pCin-LMP1 (black bars). The empty vector pCi-neo was added to equalize DNA quantities in all experiments. The luciferase activity induced by the pCin-Luc vector in the presence of 4 μg of the pCi-neo empty vector was fixed as 100% activity of the IE-CMV promoter and used to calculate relative activity of this promoter in the presence of increasing amounts of pEGFP-N3 or pCin-LMP1. (B) Similar experiment as in panel A except that the relative IE-CMV promoter activity is the percentage luciferase activity induced by the pCin-Luc vector in the presence of the indicated amounts of the pCin-LMP1 vector as compared to luciferase activity in the presence of identical amounts of the control pEGFP-N3 vector, considered as 100%. (C) Similar experiment as in panel B but with the pEF-Luc reporter vector instead of pCin-Luc in order to test the effect of LMP1 on the activity of the EF-1α promoter. (D) Similar experiment as in panel B but with the pGL3 reporter plasmid instead of pCin-Luc in order to test the effect of LMP1 on the activity of the E-SV40 promoter. (E) Activation of the NF-κB pathway measured by the increase in the quantity of luciferase produced in 10^6 DG75 cells cotransfected with 2 μg pNF-κB-Luc reporter plasmid and 4 μg of the indicated vectors. (F) Western blot detection of LMP1 in DG75 cells transfected with the indicated amounts of the pCin-LMP1 vector.

that transfection of increasing amounts of pCin-LMP1 plasmid effectively resulted in increasing amounts of LMP1 in the cells (Fig. 1F). To rule out the unlikely possibility that LMP1 could directly interfere with luciferase to block its activity, similar experiments were also performed using the pCin-βgal vector (containing the β-galactosidase gene as a reporter instead of the luciferase gene) and identical results were obtained (results not shown). All these results indicate that LMP1 is able to specifically inhibit gene expression from several different but not all promoters. As the IE-CMV, EF-1α and GAPDH promoters were similarly inhibited by LMP1, only one promoter was analyzed in most of the following experiments, which were performed with the pCin-Luc reporter.

LMP1 inhibits the activity of the endogenous EF-1α and GAPDH promoters

In order to check the relevance of these transfection experiments, we tested the effect of LMP1 expression on the endogenous EF-1α and GAPDH gene promoters. DG75 cells were cotransfected with pSVK3-EGFP (a vector driving expression of the GFP under the control of the E-SV40 promoter shown to be insensitive to LMP1-induced inhibition; Fig. 1D) in addition to pCin-LMP1 or pCin-β gal. Forty hours later, cells were sorted according to their GFP status using an Epics Altra cell sorter. RNA was extracted from GFP-positive populations. After cDNA synthesis, we checked by RT-PCR that the BNLF1 gene was expressed only in cells transfected with pCin-LMP1 and positive for GFP (results not shown). Then, using a semi-quantitative RT-PCR assay, the levels of EF-1α and GADPH mRNA were compared in β-galactosidase- and in LMP1-positive cells. As shown in Fig. 2, EF-1α and GAPDH mRNA levels were slightly albeit significantly decreased in LMP1-expressing cells as compared to β-galactosidase-expressing cells.

The carboxy-terminal domain is dispensable for the inhibitory activity of LMP1

To test whether this inhibition was dependent on signaling pathways classically activated by LMP1, we performed similar experiments with an LMP1 derivative corresponding to the first 190 amino acids of the wt protein and thus lacking the complete intracytoplasmic C-terminal domain (ΔCT-LMP1). As negative and positive controls, we used the β-galactosidase and the wt BNLF1 genes, and results show that LMP1 and its ΔCT-LMP1 derivative inhibit the IE-CMV promoter activity with equal efficacy (Fig. 3A), indicating that the LMP1 amino-terminal and TM domains alone suffice to confer complete inhibitory capacity to the protein.

LMP1 inhibits the IE-CMV promoter activity through its transmembrane domains 3–4 or 5–6

Several groups have reported that all TM domains of LMP1 are not equivalent and that the TM domains 1–2 are particularly implicated in lipid raft targeting (Yasui et al., 2004). This prompted us to try to map the region of LMP1 involved in this inhibition. For this purpose, we generated a series of mutants lacking one pair of TM domains but retaining normal amino- and carboxy-terminal extremities: N-TM3456-C, lacking TM domains 1 and 2 (corresponding to amino acids 24 to 74), N-TM1256-C, lacking amino acids 75 to 133, and N-TM1234-C, lacking amino acids 133 to 190. Cotransfections of these mutants in DG75 cells with the pCin-Luc reporter plasmid showed that they all retained the full inhibitory capacity of the wt LMP1, suggesting that this inhibitory capacity is not associated with a single pair of TM domains (Fig. 3B). We then constructed a second series of similar LMP1 derivatives, but with each retaining only one single pair of TM domains: N-TM12-C, lacking TM domains 3, 4, 5 and 6 (from amino acids

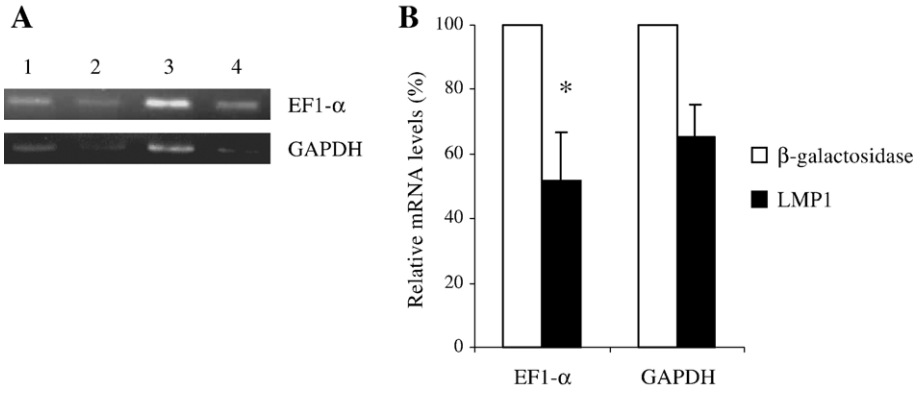

Fig. 2. Effects of LMP1 on the EF-1α and the GAPDH endogenous promoters. Ten independent electroporations were performed on one million DG75 cells with 2 μg of pSVK3-EGFP vector and 2 μg of pCin-βgal or pCin-LMP1 vectors. Forty hours after transfection, cells were sorted according to GFP expression. RNAs were extracted from each population, converted into cDNAs which were used as templates in semi-quantitative RT-PCR assays targeting EF-1α or GAPDH sequences. Amplification products were analyzed on 2% agarose gels and quantified using the GeneTools software (Syngene). (A) EF-1α or GAPDH semi-quantitative RT-PCR performed on cDNA prepared from GFP-positive cells expressing β-galactosidase (lanes 1 and 3) or LMP1 (lanes 2 and 4). Amplification products were obtained after 14 RT-PCR cycles (lanes 1 and 2) or 16 RT-PCR cycles (lanes 3 and 4). The results are representative of two (GAPDH) or three (EF-1α) independent experiments. (B) Quantification of GAPDH and EF-1α RT-PCR products from GFP-positive, β-galactosidase-expressing cells (considered as 100%, open bars) and GFP-positive, LMP1-expressing cells (black bars). Analysis was performed in both cases on RT-PCR products obtained after 14 cycles. Presented values are averages of two (GAPDH) or three (EF-1α) independent experiments (*$P < 0.05$ by Student's t test).

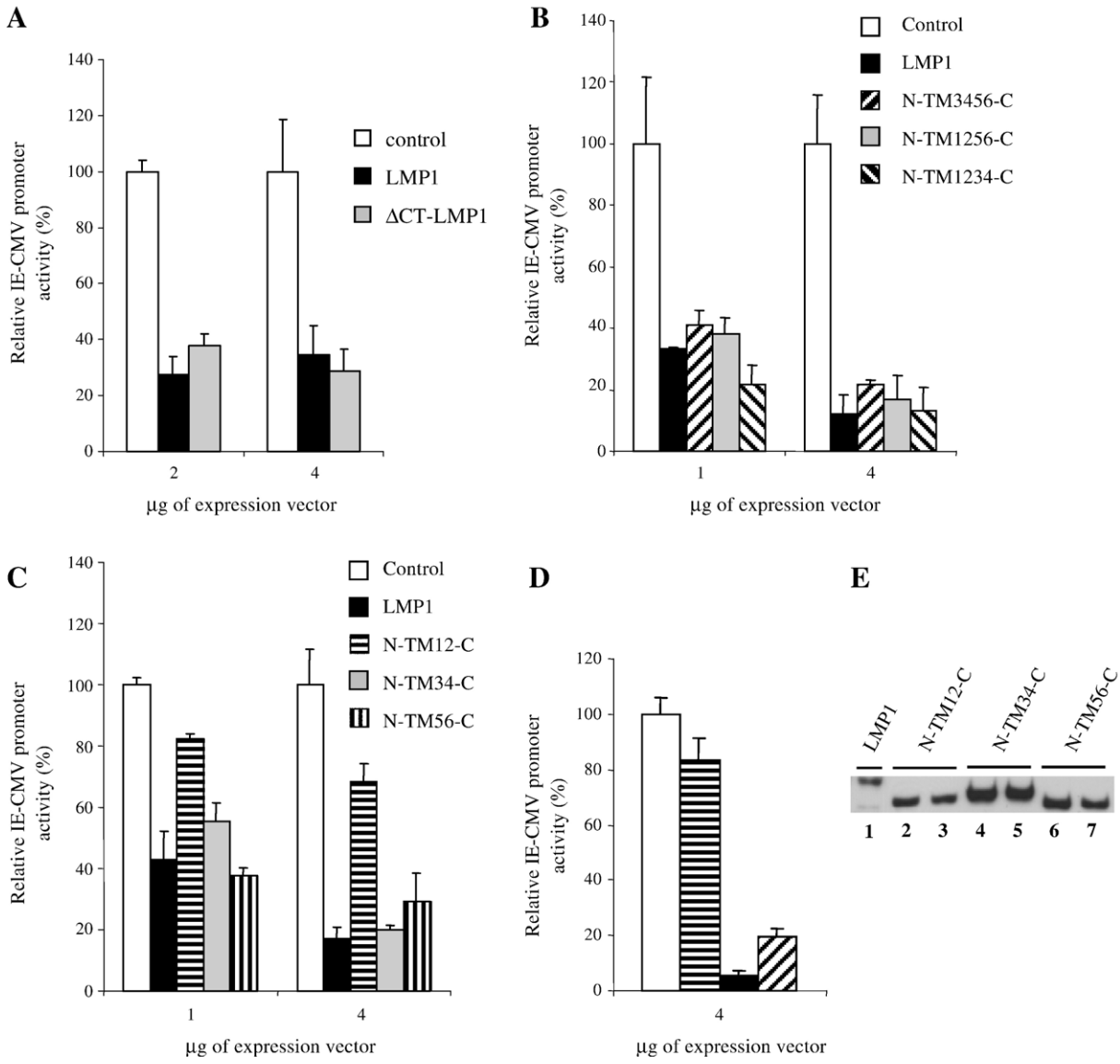

Fig. 3. Inhibition of the IE-CMV promoter by an LMP1 mutant lacking the carboxy-terminal region and identification of the membrane-spanning domains involved in the LMP1-induced inhibition of the IE-CMV promoter activity. (A) One million DG75 cells were electroporated with 2 µg of the pCin-Luc reporter plasmid and the indicated amounts of plasmids expressing a control gene (β-galactosidase, open bars), the wild-type LMP1 (black bars) or its ΔCT derivative (grey bars). Twenty-four hours after transfection, cells were lysed and luciferase quantified. The relative IE-CMV promoter activity is expressed as the percentage of luciferase activity detected in the presence of LMP1 or its DCT-LMP1 derivative as compared to luciferase activity produced in the presence of identical quantities of the control vector, considered as 100%. (B) One million DG75 cells were electroporated in the presence of 2 µg of the pCin-Luc reporter plasmid and 1 or 4 µg of pCin-βgal vector as the control, pCin-LMP1 or LMP1 mutants lacking one pair of TM domains: N-TM3456-C, N-TM1256-C and N-TM1234-C. The relative IE-CMV promoter activity was calculated as in panel A. (C) Similar experiment as in panel B excepted that LMP1 mutants with a single pair of TM domains were tested: N-TM12-C, N-TM34-C and N-TM56-C. (D) Similar experiment as in panels B and C except that electroporations were performed in the presence of pSVK3-EGFP and that results were normalized on the percentages of GFP positive cells in each transfection. Effects of N-TM12-C (horizontal stripes), full-length LMP1 (black bars) and N-TM3456-C (oblique stripes) were compared to the β-galactosidase control (empty bars). (E) Western blot analysis of LMP1 or derivatives expression in the lysates of cells transfected with 4 µg of plasmids and used to generate the results presented in panel C. Detection was performed using the S12 anti-LMP1 monoclonal antibody.

75 to 190); N-TM34-C, lacking TM domains 1–2 and 5–6 (amino acids 24 to 74 and 133 to 190) and N-TM56-C, lacking amino acids 24 to 133. When analyzed for their ability to inhibit luciferase expression from the pCin-Luc vector, these mutants revealed different behaviors: while N-TM34-C and N-TM56-C seemed to inhibit as well as the wt LMP1, N-TM12-C appeared significantly impaired in its ability to inhibit luciferase production from the pCin-Luc vector (Fig. 3C). A similar experiment was again performed with wt LMP1 or with mutants N-TM3456-C and N-TM12-C, but including the pSVK3-EGFP vector in order to normalize the results on the fractions of GFP-positive cells. As shown in Fig. 3D, results are nearly identical to those obtained without normalizing on GFP expression (Fig. 3C). We verified expression of the different mutants in parallel with the lysates used for luciferase expression assays and all appeared to be expressed at similar or higher levels than the wt LMP1, suggesting that the observed difference in inhibitory capabilities between the different mutants could not be

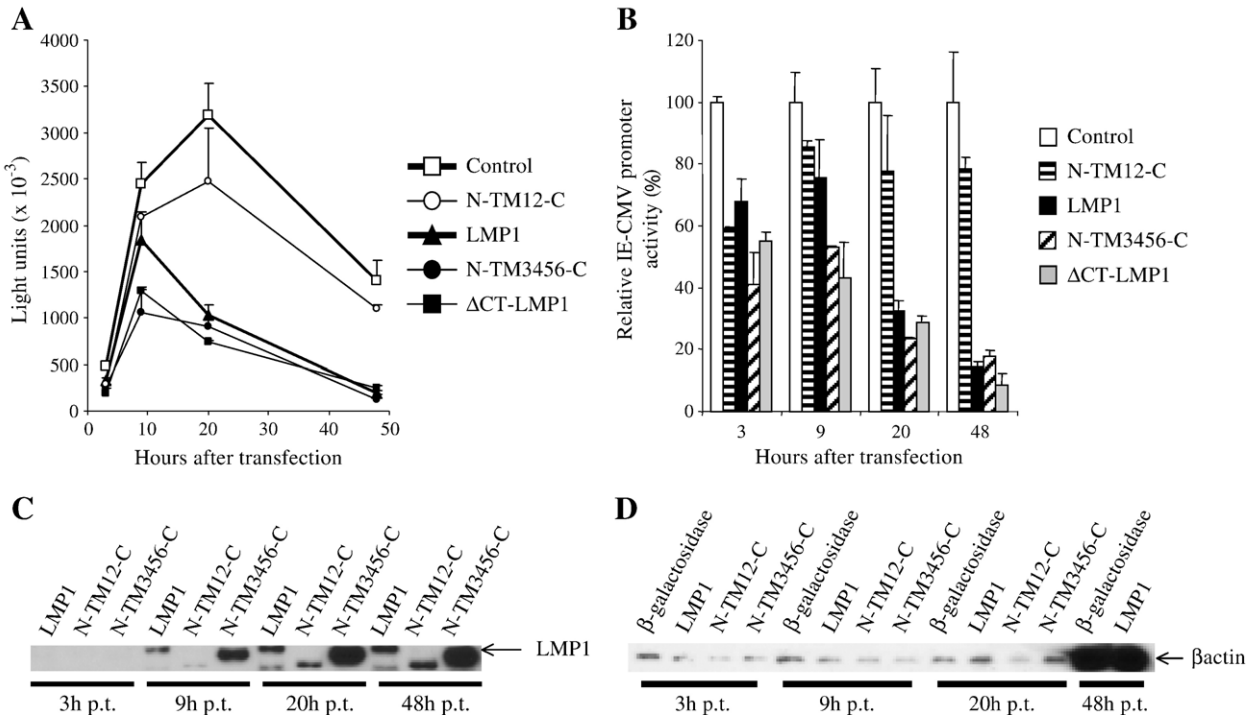

Fig. 4. Kinetics of the LMP1-induced inhibition of the IE-CMV. Three aliquots of 10^6 DG75 cells were electroporated with 2 μg of the pCin-Luc reporter plasmid and 2 μg of the indicated plasmids, subsequently pooled and cultivated together. Duplicate aliquots corresponding to 2.5×10^5 initial cells were harvested 3, 9, 20 and 48 h after transfection, lysed in 100 μL 1× CCLR and analyzed for luciferase activity as described. (A) Time course analysis of the luciferase activity present in the lysates of cells transfected with the indicated plasmids, expressed as light units. (B) Same as panel A but expressed as relative IE-CMV promoter activity calculated by comparison with the luciferase activity in the presence of the pCin-βgal control plasmid, considered as 100% activity at each time point. (C) Western blot analysis of LMP1 or derivative expression (excepted ΔCT-LMP1) in the lysates used to generate the results presented in panels A and B. Detection was performed as in Fig. 3C. (D) Western blot analysis as in panel C but revealed with the AC15 anti-β-actin monoclonal antibody.

explained by differential expression (Fig. 3E). These results strongly suggest that molecules containing normal amino- and carboxy-terminal domains and either the TM segments 3–4 or the TM segments 5–6 retain complete inhibitory capacity, while the TM domains 1 and 2 do not seem to be significantly involved in this activity.

Kinetic analysis of the LMP1 inhibitory activity

In order to characterize the kinetics of this inhibition, we cotransfected in DG75 cells the pCin-Luc reporter plasmid with expression vectors for the β-galactosidase gene, for LMP1 or for its ΔCT-LMP1, N-TM3456-C or N-TM12-C derivatives. Aliquots from the different experiments were taken after various periods of time and analyzed for expression of luciferase and LMP1 or its derivatives. As shown in Figs. 4A and B, inhibition of luciferase production by LMP1 and its inhibitory derivatives was observed as early as 9 h post-transfection. Moreover, luciferase activity increased up to 20 h in cells cotransfected with the β-galactosidase gene or the N-TM12-C mutant, but displayed a marked decrease at this time point in cells expressing the wt LMP1 or its inhibitory derivatives. The difference in luciferase activity was still evident 48 h after transfection when cells resumed protein synthesis and likely proliferation as shown by a huge increase in actin synthesis at this time. Western blot analysis showed that LMP1 and its derivatives were not all expressed at identical levels during the course of this experiment (Figs. 4C and D). N-TM3456-C in particular showed much higher expression than the others. However, N-TM12-C and the wt LMP1 were expressed at similar levels (at least from the 20 h time point), suggesting that their different behaviors were not the consequence of differential expression.

Inhibition of gene expression does not induce cytostasis

Kaykas et al. have published that expression of an LMP1 derivative containing only the amino-terminal and the membrane-spanning domains induced cytostasis. To investigate a possible relationship between the herein described inhibition of different promoters and cytostasis, we decided to follow the proliferation of DG75 cells transfected with either the β-galactosidase gene, LMP1, or various derivatives, using the Cell Tracker Orange™ CMTMR. This fluorescent dye irreversibly binds to thiol groups inside the cell and is diluted upon cell division, allowing cytofluorimetric monitoring of cell proliferation. As controls for proliferating and nonproliferating cells, we used electroporated, CMTMR-stained cells cultivated in normal medium or in the presence of mitomycin C, which blocks cell division. As shown in Fig. 5A, CMTMR labeling decreased only in untreated, dividing cells. In order to separate transfected from nontransfected cells in these experiments, we

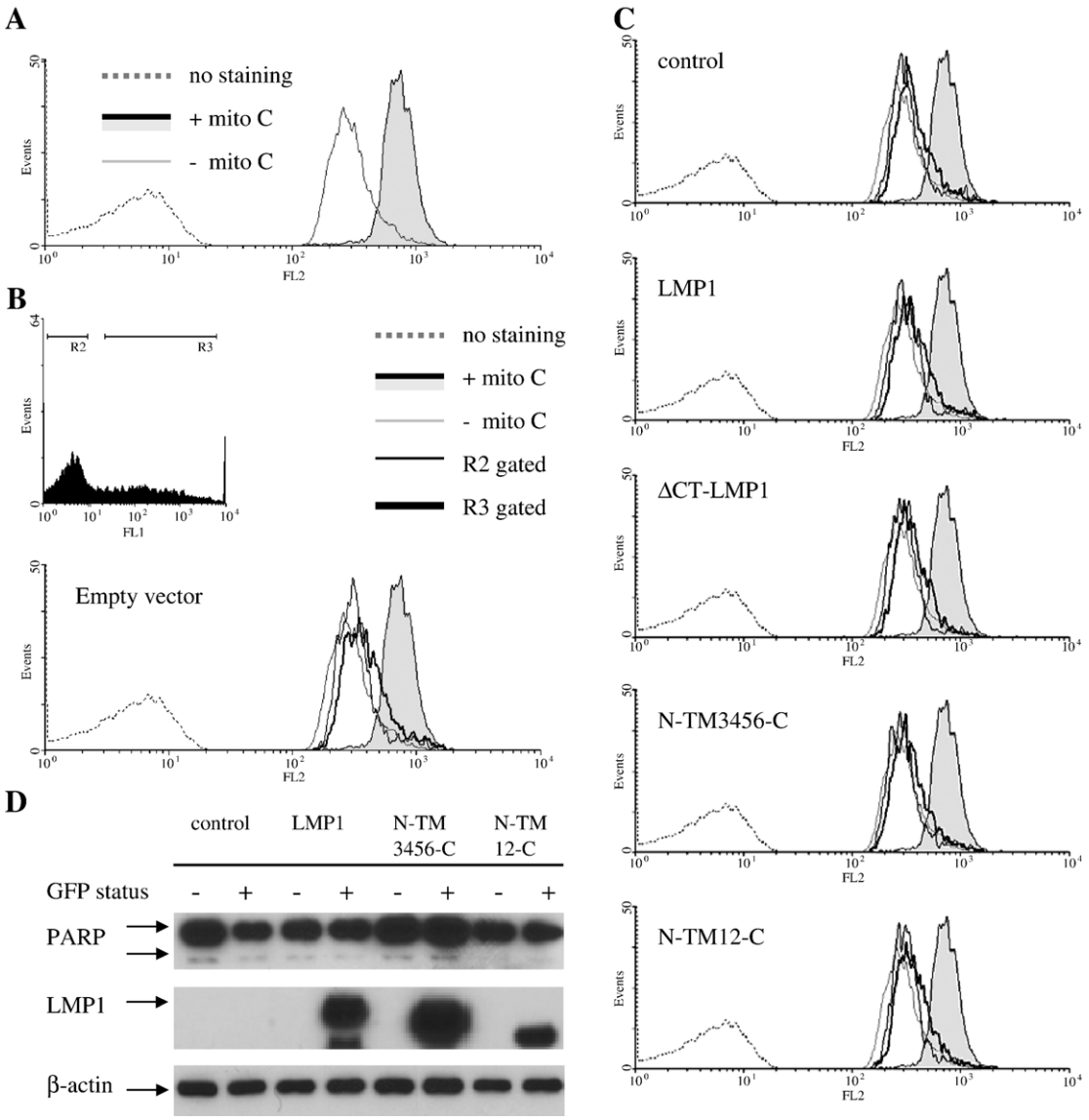

Fig. 5. Effect of LMP1 or derivatives on DG75 cell proliferation and apoptosis. (A) One million DG75 cells were stained with CMTMR Cell Tracker Orange (Molecular Probes), electroporated without adding any plasmid and cultivated in normal medium (solid grey line) or in medium containing 10 μg/mL mitomycin C to block cell division (solid black line, filled histogram). Forty-eight hours later, cells were analyzed for CMTMR fluorescence using an Epics XL4C FACS. Decrease of the fluorescence in the FL2 channel indicates that cells not treated with mitomycin C normally divide, when mitomycin C treated cells do not. These populations are used as proliferating (solid grey line) and nonproliferating (solid black line, filled histogram) controls for the next experiments together with nonstained cells (dotted line). (B) One million DG75 cells were stained with CMTMR Cell Tracker Orange, electroporated with 4 μg pSVK3-EGFP and 2 μg pCi-neo plasmids and analyzed 48 h later for GFP fluorescence (FL1). CMTMR labeling (FL2) was subsequently analyzed on GFP positive (R3, black bold line) and GFP negative (R2, black thin line). (C) Similar experiment as in panel B except that the pCi-neo empty vector was replaced by the indicated vectors expressing β-galactosidase, LMP1, ΔCT-LMP1, N-TM3456-C or N-TM12-C mutants. (D) Equal quantities (2×10^5) of GFP-positive and -negative cells from the experiments described in panel C were purified using a High Speed cell sorter Epics Altra, lysed and analyzed by Western blot for LMP1 or derivative expression, for β-actin and for the cleavage of PARP using, respectively S12, AC-15 and C2-10 monoclonal antibodies.

cotransfected the different LMP1 constructions with the pSVK3-EGFP plasmid, bringing the GFP expression under the control of the LMP1-unsensitive E-SV40 promoter. Forty-eight hours after transfection, GFP-positive and -negative cells were analyzed by FACS for CMTMR fluorescence. As shown in Fig. 5B, when cells were electroporated in the presence of pSVK3-EGFP and the empty pCi-neo vector, the GFP-positive and -negative cells divided at the same rate as the control cells not treated with mitomycin C. Fig. 5C illustrates the proliferation of GFP positive cells expressing β-galactosidase, LMP1, ΔCT-LMP1, N-TM3456-C or N-TM12-C derivatives, as compared to the corresponding GFP-negative cells from each experiment. These results strongly suggest that expression of LMP1 or its inhibitory derivatives has no effect on cell division and does not induce cytostasis in this cellular model. In order to validate this approach and the fact that the GFP-positive cells truly were the cells transfected with the tested plasmids, we sorted the GFP-positive and -negative cells from the same experiments using the Epics Altra cell sorter. The same number of cells from both populations was lysed and analyzed in

Western blot experiments for the presence of wt LMP1 or derivatives. As shown in Fig. 5D, large quantities of LMP1 or derivatives were detected only in GFP-positive cells, and no signal was observed with the S12 anti-LMP1 antibody in the GFP-negative cells, confirming that, in these experiments, LMP1 or derivatives were expressed at detectable levels only in the GFP positive cell population.

LMP1 expression does not induce apoptosis

As it has been reported that expression of large quantities of LMP1 could be toxic for cells (Hammerschmidt et al., 1989), it was of interest to observe the apoptosis status of our LMP1 transfected cells. It is well documented that the cleavage of the poly-(ADP-Ribose)-polymerase (PARP) p115 isoform is an early event in apoptosis (Patel et al., 1996). Therefore, we analyzed this cleavage in the GFP-positive and -negative cell populations obtained from the sorting. As can be seen in Fig. 5D, the greatest quantity of PARP remained in the p115 isoform and only minute amounts of low MW PARP isoform could be detected in all lysates, regardless of whether they originated from GFP-positive or -negative cells and whatever the LMP1 protein expressed. It is therefore very unlikely that LMP1 or any of the derivatives we tested in this study were able to induce significant apoptosis in this cellular model under the conditions we used.

Discussion

In this study, we report that gene expression from different natural complex promoters is inhibited in the presence of the EBV oncoprotein LMP1 in DG75 human B cells. This effect was mainly studied on the IE-CMV promoter, which is considered constitutive and contains NF-κB and AP-1 response elements, but was also observed on the cellular promoters of the EF-1α and GAPDH genes, which are differently regulated. On the contrary, the E-SV40 promoter, which is also sensitive to NF-κB, completely eluded this LMP1-induced inhibition indicating that it is not a general phenomenon. Sandberg et al. have already published similar results, but on EBV gene promoters, namely the *Bam-Cp* and the BNLF1 gene promoter. We thus extend their observations to another herpes virus promoter (IE-CMV) and to cellular promoters, suggesting that this inhibition could hit enough cellular promoters to induce the reported decrease in protein synthesis (Sandberg et al., 2000). An LMP1 mutant deleted of the complete C-terminal region retained the same inhibitory capacity as the wt protein, indicating that this phenomenon was not dependent on the already described signaling pathways activated by LMP1, but conversely required the membrane-spanning domains and likely involved new pathways. Using a series of mutants lacking one or two pairs of TM domains, we were able to map more precisely than ever before the inhibitory regions of LMP1 to the TM domains 3–4 and 5–6, each of these pairs of TM domains in association with normal amino- and carboxy-terminal domains being sufficient to achieve inhibition similar to that observed with the wt protein. Conversely, an otherwise normal LMP1 molecule containing only the TM segments 1 and 2 loses most of its inhibitory potential. Interestingly, the N-TM12-C mutant was still able to activate the NF-κB pathway (although less efficiently than the wt protein), indicating that it was indeed addressed to membranes in which it was inserted correctly enough to mediate NF-κB activation (results not shown and Coffin et al., 2003). It is noteworthy that TM domains 1 and 2 appear of particular importance among the TM segments as the TM segment 1 contains the FWLY motif described to be necessary for lipid raft targeting (Yasui et al., 2004). Moreover, an LMP1 derivative lacking the TM domains 3, 4, 5 and 6 retains a significant ability to form signaling complexes, which is not observed with mutants lacking the TM segments 1 and 2 (Coffin et al., 2003).

The fact that the TM domains 1 and 2 do not contribute to the inhibitory activity of the protein, which is mainly mediated by TM domains 3, 4, 5 and 6, is a supplementary proof that the TM domains are not all functionally equivalent but rather mediate specialized functions. Interestingly, when comparing the protein sequences of TM domain pairs 1–2, 3–4 and 5–6, they all appeared equally divergent from each other with identities of 28.6%, 21.6% and 29.6% between pairs 1–2/3–4, 1–2/5–6 and 3–4/5–6, respectively. However, comparison of TM domains 3 and 5 revealed the presence of a similar LLL/IIALW/Y motif in both sequences. We speculate that this motif is involved in the inhibitory capacity of the LMP1 protein and we are currently investigating this possibility.

Higuchi et al. have shown that D1LMP1, which is naturally initiated on the methionine 129 and contains only a short intracytoplasmic segment, the TM domains 5 and 6 and the complete C-terminal region, was not present in the lipid rafts, even in the presence of the wt LMP1 (Higuchi et al., 2001). As a consequence, it appears that D1LMP1 very poorly (or not at all) associates with the full-length LMP1. Similar results were also published by Gires et al. (1997) and Yasui et al. (2004). As a consequence, it is highly probable that our N-TM56-C mutant, which retains the full inhibitory capacity of the LMP1 wt protein, does not oligomerize and is not present in the rafts, suggesting that it is able to exert its inhibitory effect on various promoters as a monomer and without requiring particular localization. Conversely, it would be of interest to determine whether this inhibitory capacity is only characteristic of monomeric LMP1 or whether it is also exhibited by the oligomerized protein.

Surprisingly, the inhibitory effect of LMP1 and its derivatives on the IE-CMV promoter takes hold very rapidly and is already evident as soon as 9 h after transfection. At this time point, only minute amounts of proteins expressed from the transfected vectors are detectable, but these quantities are already sufficient to exert their inhibitory effect as compared to the noninhibitory derivatives. These results suggest that this inhibition is likely highly specific and does not depend on competition for transcription and/or translation factors, but is rather an intrinsic property of the molecule that is effective at low concentration and as soon as it is produced. Furthermore, our results presented in Figs. 1B and C show that LMP1 inhibits gene expression in a dose-dependent manner. However,

Hammerschmidt et al. (1989) have reported that strong expression of LMP1, driven by the IE-CMV promoter, induces significant toxicity, at least in rodent cells. As all our LMP1 constructions were expressed under the control of the strong IE-CMV promoter, it was important to rule out the trivial possibility that LMP1-induced inhibition could simply translate massive cell death due to apoptosis. This was unlikely in our experiments, as the E-SV40 promoter was resistant to the inhibitory effect of LMP1 doses which induced nearly complete inhibition of IE-CMV, EF-1α or GAPDH promoters in the same DG75 cells. Moreover, we observed that DG75 cells positive for LMP1 or its inhibitory derivatives did not show any increase in the cleavage of the poly-(ADP-ribose) polymerase (PARP), as compared to LMP1-negative cells from the same transfection experiment (Fig. 5D). As this cleavage is a sensitive and early marker of apoptosis (Patel et al., 1996), these results exclude apoptosis as an explanation for the nearly complete inhibitory effect of LMP1 on various promoters.

Different groups have reported that expression of LMP1 or its ΔCT derivative induces an arrest in cell growth. Coffin et al. (2003) also reported that the TM domains 1 and 2 are responsible for this observed cytostasis. Surprisingly enough, in our hands, neither LMP1 nor any of its derivatives induced cytostasis in DG75 cells. The reasons for these discrepancies are not clear, but we find it difficult to conceive that LMP1-induced cytostasis could be a general phenomenon. Firstly, LMP1 expression has been widely demonstrated in EBV-immortalized B lymphocytes (LCL), where it is even required to keep them growing (Kaye et al., 1993). Secondly, numerous EBV-associated tumors, including Hodgkin's disease and nasopharyngeal carcinoma, display LMP1 expression (Kieff and Rickinson, 2001). Moreover, Kilger et al. reported that in their experimental system based on conditional LMP1 expression in B lymphocytes immortalized with mini EBV plasmids, LMP1 expression was required for cell proliferation (Kilger et al., 1998). Possible explanations to these divergent results are not straightforward but could perhaps be found in the use of different techniques to evaluate cytostasis. For instance, Kaykas and Sugden cotransfected expression plasmids for LMP1 or its derivatives and for the β-galactosidase reporter gene, and monitored the evolution of the transfected population as a function of the β-galactosidase-positive cells. A decrease in the proportion of β-galactosidase positive cells was considered evidence of LMP1-induced cytostasis in transfected cells (Kaykas and Sugden, 2000). The results we report herein allow a new interpretation of these experiments, in which the β-galactosidase gene was under the control of the IE-CMV promoter. Therefore, as LMP1 and its so-called cytostatic derivatives strongly inhibit this promoter, their expression should markedly decrease the number of β-galactosidase positive cells, without indicating cytostasis. Cytostasis was also observed in Hep2 cells transfected with LMP1 or its ΔCT mutant and plated in the presence of selection (Hygromycin B) to which they became resistant due to the hygromycin phosphotransferase gene carried by the LMP1 expression vector. If the TK promoter driving the expression of the resistance gene is also sensitive to the LMP1-induced inhibition, the LMP1-positive cells will grow much more slowly (or not at all) in selective medium. More difficult to understand are the results of Floettmann et al. who describe the cytostatic effect mediated by inducible LMP1 expression in DG75 cells. However, their experiments were also performed under selective pressure, possibly introducing some not yet identified bias, and their LMP1-induced cytostasis was only transient.

Whatever the reasons for these opposite results, our present observations show that LMP1-induced cytostasis and LMP1-induced promoter inhibition are distinct mechanisms which do not necessarily act simultaneously. One possibility to reconcile divergent results could be to hypothesize that LMP1-induced inhibition is the consequence of at least two different and additive mechanisms. The first one is the mechanism described by Kaykas et al. and Sandberg et al., which works at high LMP1 concentration and requires TM domains 1 and 2. The second is described herein and is effective at low LMP1 concentration through TM domains 3–4 and 5–6 but not 1–2. These two mechanisms possibly act at different levels as the first one results in phosphorylation of the eIF2 α factor (Lam et al., 2004) and likely inhibition of protein synthesis while the other seems directly active at the transcriptional level. The major question now concerns the mechanisms by which LMP1, located in cell membranes, exerts its inhibition on promoters. Results obtained with ΔCT-LMP1 indicate that the signaling pathways classically described as activated by LMP1 are not involved. This rules out a simple hypothesis based on competition between different signaling pathways for limiting factors like p300, as described by Horvai et al. (1997). However, it is highly probable that LMP1 has not yet betrayed all its secrets, and Puls et al. (1999) have recently demonstrated that LMP1 could activate the small GTPase Cdc42 and thus possibly the Rho pathway downstream. Surprisingly, this activation was quite efficiently mediated by a ΔCT-LMP1 mutant, indicating that it was independent of LMP1 interaction with TRADD and TRAFs factors normally associated with TNF-receptor and other members of its family. The similarity of the LMP1 regions required for gene expression inhibition and Cdc42 activation prompts us to speculate that the inhibitory effect of LMP1 is mediated by the Rho pathway and we are currently investigating this hypothesis.

Still, the reasons why EBV has developed an LMP1 molecule endowed with such complex and divergent properties remain obscure. On one hand, LMP1 expression triggers numerous signaling pathways, leading to the activation of various important transcription factors. On the other hand, the same LMP1 under similar conditions inhibits gene expression from various, but not all, promoters. Interestingly, when effective, this inhibition appears to be dominant over activation. Indeed, the IE-CMV promoter is inhibited by LMP1 when it contains several elements responsive to NF-κB, a transcription factor strongly activated by LMP1. The results of Sandberg et al. suggest that the reasons for this complexity are not to be found in differential and advantageous expression of viral versus cellular genes, as the *Bam-Cp* and the BNLF1 gene promoter, which control genes whose expression is indispensable for latency, are

strongly inhibited by LMP1. Another, and possibly more attractive explanation, is that the efficacy of LMP1 in activating different signaling pathways could lead to a situation of general activation and instability for the cell, close to apoptosis. The inhibitory effect of LMP1 TM domains could therefore have been selected in order to counterbalance this general activation process by acting as a brake on numerous viral and particularly cellular promoters. It is noteworthy that two mechanisms at least, involving three distinct regions of the protein, could be involved in the LMP1-inhibitory activity, suggesting its great importance for the virus.

In any case, the ins and outs of the LMP1 induced inhibition of gene expression are far from being elucidated and certainly require further investigation. However, if LMP1 expression truly requires a negative feed-back control mechanism necessary for cell survival, it could lead to the development of interesting new therapeutic approaches based on the inhibition of this mechanism, whose failure could induce apoptosis in latently infected, LMP1-expressing cells.

Material and methods

Plasmids

The expression vector for the B95-8 BNLF1 gene, pCin-LMP1, has been constructed in two steps. First, the 5′ region of the gene, containing the two first exons and the first intron, has been PCR amplified from the B95-8 DNA, between oligonucleotides P4-BN-S (5′-ACACGCGTTACTCTGACGTAGCC-3′) and IE2-BN-SXb, introducing an *Xba*I site (5′-TTCTCTAGATCTTACCAAGTAAGCACCC-3′), digested with the *Mlu*I and *Xba*I enzymes and cloned in the same sites of the pCi-neo vector (Promega), previously deleted of its *Bgl*II site: this generated pCin-E1E2. The third exon of the gene was then amplified using primers E2-LMP1-AS (5′-GGGTGCTTACTTGGTAAGATCT-3′) and LMP-17-mL (5′-ATGGTAATGTCTAGAAGTAAAGAAAGG-3′) which introduces an *Xba*I site 18 nucleotides after the stop codon. This fragment was digested with the *Bgl*II and *Xba*I enzymes and cloned into the corresponding sites of pCin-E1E2, restoring a complete BNLF1 gene and generating the pCin-LMP1 vector. The pCin-ΔCT-LMP1 vector was directly derived from pCin-LMP1 by amplifying the complete region of interest between primers pCi-T7, located upstream of the pCi-neo MCS (5′-GTACTTAATACGACTCACTATAGG-3′) and LMP27AS, starting on codon 190 (5′-TCGTTGTCCATGGTAATACATCC-3′). This fragment was then ligated to the phosphorylated linker STOP-Xba (5′-TAATCTAGATTA-3′), digested with the *Mlu*I and *Xba*I restriction enzymes and cloned into the corresponding sites of the pCi-neo vector. All the TM domains mutants were generated using a strategy (to be published elsewhere) based on the amplification of two separate fragments which are subsequently ligated to obtain the desired modified sequence. This sequence is amplified again between the two external primers and then cloned into the pCi-neo vector. The N-TM3456-C mutant was created by ligation of the two fragments amplified from the pCin-LMP1 vector between the primers pairs pCi-T7/LMP2318 (5′-AGAGGAGAGGGGGGGTCC-3′) and LMP3AS (5′-GACCTTCTCTGTCCACTTGG-3′)/cLMPXb (5′-GTCGACTCTAGAAGTAAAGAA-3′), this later overlapping the beginning of the 3′UTR of the BNLF1 gene and pCi-neo sequence with an artificial *Xba*I site in between. Fragments were ligated together after phosphorylation, the ligated product was amplified again, treated with the restriction enzymes *Mlu*I and *Xba*I and finally cloned into pCi-neo. The N-TM1256-C and N-TM1234-C mutants were similarly obtained using primers pairs pCi-T7/LMP7466 (5′-TCTTCTGAAGATAAAGATGATCAAAAT-3′) and LMP28S (5′-GGTGCCACCATCTGGCAGC-3′)/cLMPXb for the N-TM1256-C mutant and the two primers pairs pCI-T7/LMP25AS (5′-TCGCCAGAGCATCTCCAATAAG-3′) and LMP24S (5′-CACAGTGATGAACACCACCACG-3′)/cLMPXb for the N-TM1234-C mutant. The mutants containing only one pair of TM domains between normal amino and carboxy-terminal regions were similarly constructed. The N-TM12-C mutant was generated by ligation of the fragments amplified from pCinLMP1 between primers pairs pCi-T7/LMP7466 and LMP24S/cLMPXb while the N-TM56-C mutant resulted from ligation of fragments amplified with primers pairs pCi-T7/LMP2318 and LMP28S/cLMPXb. The N-TM34-C mutant was obtained in the same way but amplifications were performed using the N-TM1234-C mutant as template, between primer pairs pCi-T7/LMP2318 andLMP3AS/cLMPXb. All the amplifications were performed using the Phusion High-fidelity DNA polymerase (FINNZYMES) according to manufacturer's instructions. Constructions were thoroughly verified by DNA sequencing. The pCin-Luc vector driving luciferase expression from the IE-CMV promoter was constructed by inserting the luciferase gene excised from the p-MAM-neo-luciferase vector (Clontech) with the *Nhe*I and *Xho*I restriction enzymes into the *Nhe*I and *Sal*I sites of pCi-neo. The pEF-Luc vector was derived from pCin-Luc of which the IE-CMV promoter was removed by *Nhe*I and *Bgl*II digestion and replaced by the EF-1α promoter excised from the pEF-Bos vector (Mizushima and Nagata, 1990). This vector was digested with the *Hin*dIII enzyme, treated with T4 DNA polymerase, ligated to *Bam*HI linkers and finally digested with *Bam*HI and *Xba*I restriction enzymes. The 1.2 kpB fragment bearing the EF-1α promoter was then gel purified and cloned into the *Nhe*I–*Bgl*II digested pCin-Luc vector. The pSVK3-EGFP plasmid was obtained by inserting the *Sma*I–*Xho*I EGFP fragment isolated from the pIRES-EGFP vector (Clontech) into the identical sites of the pSVK3 vector (Pharmacia). The pNF-κB-Luc vector was derived from the pNF-κB-d2EGFP (Clontech) by replacing the EGFP gene by the luciferase gene from the pMAM-neo-luciferase vector (Clontech). The *Xba*I site of pNF-kB-d2EGFP was transformed in an *Xho*I site using linkers and the plasmid was digested with the *Hin*dIII and *Xho*I restriction enzymes and gel purified.

Similarly, the *Nhe*I site of the pMAM-neo-luciferase vector was replaced by an *Hin*dIII site before digestion with the *Hin*dIII and *Xho*I enzymes. The 1.9 kpB fragment containing the luciferase gene has then been gel purified and finally cloned into the *Hin*dIII–*Xho*I-digested pNF-κB plasmid to

generate the pNF-κB-Luc reporter vector. To generate the pCin-β Gal vector, the *Hin*dIII site of the pSV-β-galactosidase vector (Promega) was first replaced by an *Nhe*I site using linkers. The β-galactosidase containing fragment was then excised using *Nhe*I and *Xba*I restriction enzymes, gel purified and ligated into the same sites of the pCi-neo vector. Correct orientation was verified by restriction mapping and DNA sequencing, and expression of β-galactosidase was controlled using the β-galactosidase enzyme assay system (Promega).

Cell lines, culture and transfections

DG75 is an EBV free established cell line derived from a Burkitt-like malignant lymphoma (Ben-Bassat et al., 1977). Cells were cultivated at 37 °C, under 5%CO_2, in RPMI1640 supplemented with 3% FBS, antibiotics and sodium pyruvate.

For transfections, DG-75 cells were centrifuged and resuspended at a concentration of 10^7 cells/mL in RPMI1640. One hundred microliters of the cell suspension was mixed with reporter and/or tested plasmids, and empty vector (pCi-neo) added to equalize DNA quantities (6 μg per 100 μL cell suspension). The mixture was transferred in a 2 mm electroporation cuvette (Eurogentec) and shocked using a Gene Pulser II electroporator (BioRad) with the following settings: R = max, C = 500 μF, V = 150 V. After electroporation, cells were cultivated in complete medium at 37 °C/5%CO2 for 24 h, unless otherwise stated.

Analysis of luciferase activity

Transfected cells were prepared according to the Luciferase Assay System Protocol (Promega) and lysed in 150 μL of 1× Cell Culture Lysis Reagent (CCLR, Promega). After centrifugation to pellet cellular debris, 20 μL supernatant was transferred into a 96 well opaque plate. Produced luciferase was quantified by measuring the light emitted upon injection of 80 μL of luciferin solution (Promega) using a Berthold Mithras luminometer. In experiments where the results were normalized according to the fractions of GFP-positive cells, half of the transfected cells were taken for FACS analysis while the rest was treated for luciferase assay.

cDNA synthesis and semi-quantitative RT-PCR

DG75 cells were cotransfected as described above with pSVK3-EGFP and pCin-β gal or pCin-LMP1 plasmids. Forty hours later, similar amounts of GFP-positive and GFP-negative cells were sorted on an Epics Altra cell sorter (Beckman-Coulter). RNA was purified using an RNeasy Mini Kit (Qiagen), ethanol precipitated and finally resuspended in RNAse free water (10 μL per 5 10^4 cells). cDNA synthesis was performed on 300 ng total RNA after olido-dT annealing, using the SuperScript Preamplification system (Gibco). For semi-quantitative RT-PCR, 5 identical tubes for each cDNA were prepared, containing 0.5 μL cDNA and 19.5 μL of the mixture: 4 μL 5× Polymerase buffer, 0.4 μL of 10 mM dNTP solution, 0.8 μL of each 10 μM primer solution and 0.1 μL (0.2 units) Phusion DNA polymerase (Finnzymes). Amplification conditions were 98 °C for 30 s and then 12, 14, 16, 18 and 20 cycles at 98 °C for 12 s, 63 °C (EF-1α) or 65 °C (GAPDH) for 15 s and 72 °C for 8 s (EF-1 α) or 12 s (GAPDH). Amplification products were then analyzed on a 2% agarose gel and quantified using the Gene Tool software. Primers used for the EF-1α amplification were EF-E6E7-S (TTCACTGCTCAGGTGATTATCCTGAACC) and EF-E8E7-AS (AACAGCAAAGCGACCCAAAGGTGG), and those for GAPDH were GAPDH-S (ACCACAGTCCATGCCATCAC) and GAPDH-AS (TCCACCACCCTGTTGCTGTA).

Western blots

CCLR lysates used for luciferase quantification were diluted with an equal volume of electrophoresis sample buffer, separated in a 0.1% SDS–12% polyacrylamide gel and electrophoretically transferred to PVDF membranes (Amersham). Membranes were incubated overnight at 4 °C with TBS supplemented with 5% nonfat dry milk and 1%Tween-20. LMP1 was detected by incubating the blots sequentially with culture supernatants from S12 mAb secreting hybridoma (diluted 1 in 300) and with the HRP goat anti-mouse IgG antibody (Dako). Actin was detected with AC-15 anti-β actin affinity purified mAb (SIGMA) and the same secondary antibody. PARP isoforms were detected by sequentially incubating the membranes with the C2-10 anti-PARP monoclonal antibody (BD Pharmingen) and the HRP-Goat anti-mouse IgG antibody. Blots were revealed using the ECLplus detection kit according to manufacturer's instructions (Amersham) and CL-Xposure films (Pierce).

Flow cytometric analysis

One to five million cells were incubated for 15 min at 37 °C in 1 mL of complete RPMI1640 containing 15 μM of CMTMR cell tracker orange (Molecular probes), washed, incubated in 2 mL of complete medium for 30 min at 37 °C, centrifuged and resuspended in FCS-free RPMI1640 medium. Stained cells were immediately mock-electroporated or electroporated with 2 μg of the indicated vectors and 4 μg of pSVK3-GFP per transfection. Half of the mock-electroporated cells were cultivated with mitomycin C (SERVA) at a final concentration of 10 μg/mL. All cells were finally cultivated in complete medium for 48 h, harvested and washed in PBS. Fluorescence was immediately read using a Coulter XL FACS and data were analyzed using WinMDI software package purchased on the facs.scripps.edu/software.html web site. Flow cytometric sorting was performed using an Epics Altra cell sorter (Beckman-Coulter), on each population of stained and transfected cells, according to correct size and forward scatters and to their green fluorescence status. The same amount of GFP-positive and -negative cells (2×10^5) was sorted from all experiments, recovered in 1 mL FCS and washed twice with PBS.

Acknowledgments

We thank Claudie Offer and Helene Brun for performing all the DNA sequencing required to verify our constructions, Fatima L'Faqihi-Olive for her help in FACS analysis and cell sorting, Jerome Terrasson and Sophie Allart for their advises in anti-PARP Western blot experiment, Fabienne Meggetto for the kind gift of the S12 monoclonal antibody and Eliane Piaggio and Anne Dejean for their help in CMTMR labeling. We are also deeply indebted to Sarah Hull who greatly helped to improve the English version of this paper. SN is a recipient of an "allocation de recherche du Ministère délégué à la Recherche et aux nouvelles technologies".

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